CII. THE MECHANISM OF THE BIOLOGICAL SYNTHESIS OF ACETYLCHOLINE. II

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In our first publication [Stedman & Stedman, 1937] on this subject it was shown that the addition of sodium acetoacetate to minced brain tissue, treated with chloroform under specified conditions, caused an appreciable increase in the formation of acetylcholine in such tissue when it was incubated at 37°. Subsequent work [Stedman & Stedman, 1938] has confirmed this result, but publication of the details of our experiments has hitherto been withheld owing to the interpretation which Mann *et al.* [1938, 1, 2] have placed upon our findings, an interpretation which has necessitated an investigation of certain aspects of our work which is more detailed than that previously carried out.

Experimental methods

Ox brain has, in general, been used in this work. The brains were brought from the slaughter-house to the laboratory as quickly as possible after the slaughter of the animal. Since, under the conditions of our experiments, the formation of acetylcholine is greater in the basal ganglia than in the remainder of the brain, this material has, as far as possible, been employed. Unfortunately, however, the corpora striata, in which the formation of acetylcholine is, of the material examined, greatest, were frequently damaged or completely destroyed during the slaughter of the animal. It has therefore been necessary to use mainly a mixture of the thalami with cortical tissue from the hemispheres. The tissue so selected was first finely minced and then thoroughly ground in a mortar. 10 g. portions of the material, thus rendered as uniform as possible, were then used in the subsequent experiment. In general, each portion was first separately ground in a mortar with 5 ml. of an organic solvent containing 5 mg. of eserine base, after which additions of further substances dissolved in a small volume of saline or phosphate Ringer (*p*H 7·4) were, if necessary, made. Where required, suitable controls were prepared at the same time. After an experimental period of 2 hr. at the required temperature the material was quickly and thoroughly mixed with 50 ml. of absolute alcohol and left overnight. For the preparation of an extract, suitable for assay or comparison, of the acetylcholine present, the alcohol was filtered and the residue twice thoroughly washed with more of the solvent. The combined filtrate and washings were evaporated to dryness under diminished pressure at low temperature, and if, as often happened, the material could not be submitted immediately to the remaining stages of purification, the dry residue was stored in an evacuated desiccator over H_2SO_4. In any case, it was next intimately mixed with a total volume, including that used for washing purposes, of 20 ml. of 10 % trichloroacetic acid, centrifuged to remove insoluble material, and the centrifugate extracted 4 times with ether to remove the bulk of the trichloroacetic acid. The solution was now made just alkaline to litmus by the addition of $NaHCO_3$, extracted 5 times with chloroform to remove the eserine, and, after again making acid with tartaric acid, evaporated to dryness under diminished pressure at low temperature. The dry residue was stored, as

before, in a desiccator. This process of purification, although long and tedious, has been used throughout this work because it not only removed a large amount of impurity from the alcoholic extracts but also yielded a product which was completely and readily soluble in water. The assay or comparison of the amount of acetylcholine in these extracts has usually been made on the dorsal muscle of the leech. Occasionally, however, strips of the small intestine of the rabbit or of the frog's auricle have been used as test objects.

Efficacy of method of extraction

Apart from the probability of its greater efficiency, the choice of alcohol rather than of aqueous solvents for the extraction of acetylcholine from brain tissue was made largely because it was thought that dilution of the tissue with a relatively large volume of a dehydrating solvent would rapidly arrest any enzymic processes which might be involved in the production of acetylcholine. Its use possessed, moreover, the additional practical advantage that the alcohol could be much more readily filtered from the residual solid than could, for example, aqueous trichloroacetic acid. It became necessary, however, to test the relative efficacies of various solvents, particularly in view of the opinions which have been expressed regarding the occurrence in nervous tissue of a bound form of acetylcholine. Such a possibility was discussed about 6 years ago by Chang & Gaddum [1933]. Since that time Corteggiani *et al.* [1936; 1937] have stated that by heating brain tissue with physiological saline in the presence of eserine at 70° for 3 min. more acetylcholine passes into the solvent than when the extraction is carried out at room temperature, and they have attributed this result to the presence in the tissue of a complex which liberates acetylcholine under the influence of heat. Loewi *et al.* [1937; 1938] have similarly found that eserinized Ringer extracts considerably less acetylcholine from the central nervous system of the frog than does $N/100$ alcoholic HCl, and have also concluded that a combined form of acetylcholine is present in nervous tissue. More recently, Mann *et al.* [1938, 1, 2] have attributed the power of chloroform, described in our first communication, of producing a large increase in the amount of extractable acetylcholine present in brain tissue to the ability of this solvent to liberate acetylcholine from a complex in which it already exists in a preformed but combined condition. The properties ascribed by the last-mentioned authors to their hypothetical complex are of such a nature that it is impossible directly to disprove its existence. It can, however, be readily shown that such a complex, if it does exist, is quite different from that of Loewi. Thus, 10 g. of brain tissue were thoroughly ground with chloroform-eserine and kept for 2 hr. at room temperature, when 100 ml. of $N/100$ alcoholic HCl were added. Three similar portions of the same material were treated immediately with 100 ml. of alcohol, $N/100$ alcoholic HCl or 10% trichloroacetic acid respectively. Alcohol and alcoholic HCl extracted identical amounts of acetylcholine from the untreated tissue; trichloroacetic acid extracted slightly less, due probably to loss on account of difficulties in manipulation; while the tissue treated with chloroform yielded 9–10 times as much as the controls. According to Loewi, $N/100$ alcoholic HCl extracts both free and bound acetylcholine from nervous tissue. It follows that the increased yield of acetylcholine obtained after treatment with chloroform does not originate from the bound form of this substance which Loewi has demonstrated to exist in nervous tissue. The experiment shows, moreover, that alcohol, which we have consistently employed for the extraction of acetylcholine from tissue, is as efficient a solvent for this purpose as $N/100$ alcoholic HCl and is at least as efficient as 10% trichloroacetic acid. Experiments quoted

below in another connexion will also demonstrate that the amount of acetylcholine extracted by acetone is identical with that obtained with alcohol. The virtual identity of the yields obtained with these various solvents suggests that they all effect a complete extraction of acetylcholine, "bound" or free, from the tissue. This result does not appear to us to be in conformity with the view that the much larger amount of acetylcholine produced relatively slowly in the presence of chloroform is already preformed in the tissue.

Influence of temperature on the chloroform effect

When we first demonstrated the power of chloroform to increase the formation of acetylcholine in brain tissue, our experimental procedure involved the incubation of the material at 37°. No reason was given for the choice of this particular temperature, and, beyond the unverified assumption that any effect which chloroform might possess would be greater at body than at other temperatures, none, in fact, existed. Mann *et al.* [1938, 1] have, however, pointed out that the effect of chloroform is greater at room temperature than at 37°. They claim, moreover, that it is as great at 0° as at 37°. It must be confessed that if this were the case and if amounts of acetylcholine comparable with those formed at higher temperature were, in fact, liberated at 0°, the argument that such acetylcholine represented newly synthesized material would be considerably weakened. Many experiments, of which the following is typical, have therefore been carried out to test this point.

Four portions of brain tissue were separately treated with chloroform-eserine. Of these, the control was mixed immediately with alcohol, while the remainder were kept for 2 hr. at 0°, room temperature and 37° respectively. The acetylcholine present was then extracted and the yields compared on the leech muscle. The results showed that the ratios of the amounts of acetylcholine obtained were: control: 0° : 37° : room temperature = 1 : 1·4 : 5 : 6·5, from which it follows, after deducting the value of the control from the remainder, that the amounts of acetylcholine produced during the experiment were in the ratio 0° : 37° : room temperature = 1 : 10 : 13·8. It is thus clear that the amount of acetylcholine produced at 0° is only 1/14 and 1/10 of that formed at room temperature and 37° respectively. This amount, while not entirely negligible, is so small that it appears justifiable to assume that it is mainly produced during the short period necessary to reduce the temperature of the material to 0°. Similar results were obtained in other experiments, in some of which the acetylcholine was assayed on strips of rabbit's intestine.

Chloroform evidently exerts a dual action on brain tissue. In addition to facilitating the formation of acetylcholine, it also effects a destruction of the mechanism responsible for this formation. At 37° the latter process is much accelerated; hence the somewhat smaller yield of acetylcholine. That this is an adequate explanation of the phenomenon appears to be confirmed by the facts, which will be demonstrated below, that ether is superior to chloroform in its ability to cause the formation of acetylcholine in brain tissue, and that its effect is, moreover, greater at 37° than at room temperature. The destructive action of ether on the mechanism involved is apparently either smaller than that of chloroform or is entirely absent.

Effect of various organic solvents on the formation of acetylcholine

In attempting to elucidate the mechanism of the action of chloroform in increasing the amount of extractable acetylcholine in brain tissue, we have carried out a number of experiments in which chloroform has been replaced

by other solvents. These quickly demonstrated that ether is more effective both at room temperature and at 37° than chloroform under the same conditions. Experiments with acetone at room temperature, however, gave yields of acetylcholine identical with those obtained both in the usual control and in one in which the 10 g. sample of brain tissue was treated immediately with 100 ml. acetone. Alcohol, on the other hand, yielded, at room temperature, slightly more acetylcholine than the control. In two experiments, again at room temperature, a quantitative comparison was made of the yields obtained with the effective solvents. In one of these, six times as much acetylcholine was obtained from the sample treated with ether, and 3·2 times as much from that treated with chloroform, as from the one treated with alcohol. In the second the usual control was also made. The results can be summarized by the ratio control : alcohol : chloroform : ether = 1 : 1·9 : 4·1 : 7. If the amount formed in the control is deducted from the remainder, the ratio alcohol : chloroform : ether = 1 : 3·5 : 6·7 is obtained. It is apparent from these results that the two strongly dehydrating solvents, alcohol and acetone, both destroy the mechanism responsible for the formation of acetylcholine in brain tissue. Acetone is, however, evidently the more effective in this respect since some formation continues in the presence of alcohol unless relatively large volumes, such as we use in the extraction of the acetylcholine, are employed. The fact that the yield obtained from brain tissue after treatment with chloroform is less than that produced in the presence of ether can thus clearly be attributed to a similar, but less rapid, destruction of the mechanism in question by this solvent. Not only, however, does ether cause a greater formation of acetylcholine at room temperature than chloroform, but it also differs from the latter solvent in that its effect is increased at 37° whereas that of chloroform is diminished. This is apparent from Fig. 1.

Effect of various substances on the formation of acetylcholine

We have already stated [Stedman & Stedman, 1938] that the addition of sodium acetoacetate to brain tissue treated with chloroform-eserine caused an increase of 100 % in the yield of acetylcholine extractable from such tissue, and that the addition of choline produced a further, but smaller, increase in the yield. In the experiments, which have not hitherto been recorded, on which this statement was based, the brain tissue was prepared according to our standard procedure, four portions being separately treated with chloroform-eserine and then ground with 2 ml. of saline without further addition or with the same volume of saline containing 10 mg. of choline chloride, 20 mg. of sodium acetoacetate or of both these substances respectively. The material was incubated for 2 hr. at 37° when the acetylcholine was extracted, acetoacetate and/or choline in the above amounts being added to the alcoholic extracts of the samples which had not been treated with these substances, and a comparison made of the amounts obtained from the various portions. Some of the leech tracings are reproduced in Fig. 2. An examination of the responses shows that, both in the presence and absence of acetoacetate, choline causes a small but definite increase in the yield of acetylcholine. A similar and subsequent experiment, moreover, completely confirmed this result. Nevertheless, it must be recorded that in several experiments, identical with the above except that the material has been kept at room temperature instead of at 37°, we have since been unable to demonstrate any effect due to choline.

As regards the influence of acetoacetate, it is evident from Fig. 2 that this substance has approximately doubled the yield of acetylcholine. This rough

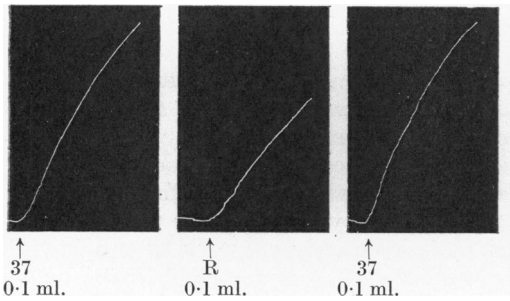

Fig. 1.

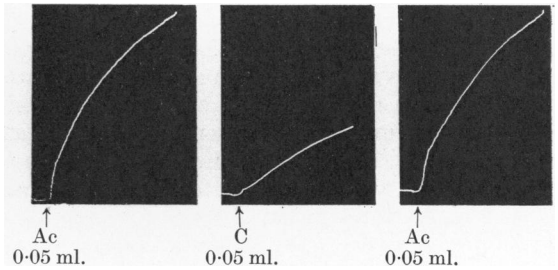

Fig. 3.

Fig. 1. Effect of temperature on the formation of acetylcholine in the ether preparation. R = kept at room temperature for 2 hr.; 37 = incubated at 37° for 2 hr. Final volume of extracts = 250 ml.

Fig. 3. Effect of acetoacetate on the formation of acetylcholine in the chloroform preparation at room temperature. Final volume of extracts = 50 ml. Ac = with added acetoacetate; C = control.

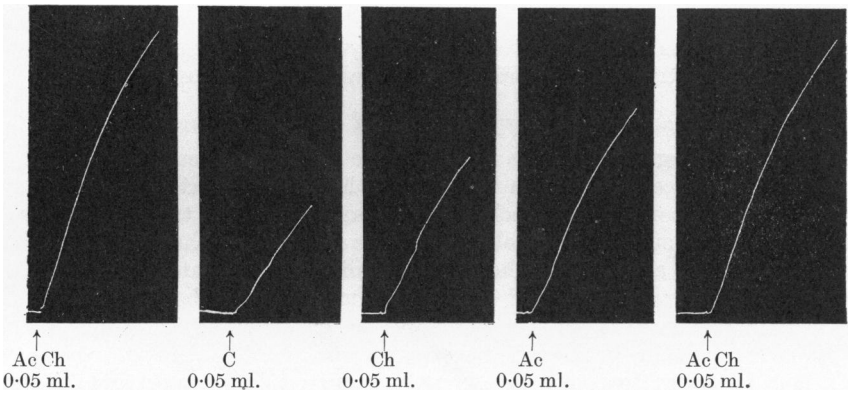

Fig. 2. Effect of sodium acetoacetate and of choline on the formation of acetylcholine in the chloroform preparation at 37°. For details, see text. Final volume of extracts = 250 ml. C = control; Ac = with added acetoacetate; Ch = with added choline; AcCh = with added acetoacetate and choline.

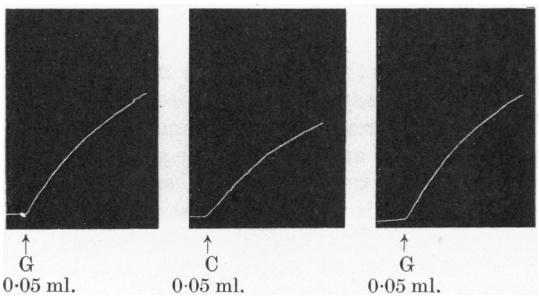

Fig. 4.

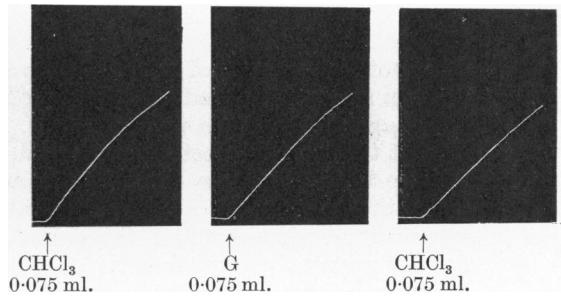

Fig. 5.

Fig. 4. Effect of glucose on the formation of acetylcholine by brain tissue suspended in phosphate-Ringer at 37°. Final volume of extracts = 15 ml. G = with added glucose; C = control.

Fig. 5. Continuation of Fig. 4, showing absence of effect on shaking with chloroform after incubation. $CHCl_3$ = shaken with chloroform.

estimate of its effect has, moreover, been confirmed in numerous similar experiments in which more accurate comparisons with the controls have been made. Mann *et al.* [1938, 1] appear also to have confirmed this result, but they state that, even in the presence of acetoacetate, the amount of acetylcholine produced at 37° is no greater than that at room temperature in its absence. They further claim that acetoacetate does not increase the yield of acetylcholine if the tissue is kept at 18° in the presence of chloroform. This is entirely contrary to our experience. Fig. 3, for example, shows that addition of acetoacetate to the chloroform preparation at room temperature greatly increases the yield of acetylcholine, and similar results have been obtained on numerous occasions. Since there is thus no doubt that acetoacetate does increase the formation of acetylcholine in such preparations, the point as to whether or no the yield at 37° in the presence of acetoacetate is no greater than at 18° in its absence becomes immaterial.

We have previously shown [Stedman & Stedman, 1937] that addition of sodium pyruvate to brain tissue treated with chloroform-eserine diminishes the formation of acetylcholine in such tissue when it is incubated at 37°. In view of the above results, the effect of this substance has been examined at room temperature. It has been found that sodium pyruvate still exerts a considerable inhibitory action on the formation of acetylcholine under these conditions.

Quantitative assays of the yield of acetylcholine

In the preceding experiments the influence of any particular treatment on the formation of acetylcholine in brain tissue has been ascertained by directly comparing the response of the leech muscle to extracts of the treated tissue with similar extracts of suitable controls. While this is probably the most rapid, and certainly the most accurate method of determining the relative effects of the treatment, it nevertheless seemed desirable to measure the absolute amounts of acetylcholine produced under certain conditions. Some results of such measurements are recorded in the following experiments.

Of five 10 g. portions of minced and ground ox brain, one (control) was treated immediately with 50 ml. alcohol, two were ground with chloroform-eserine and two with ether-eserine, 1 ml. of saline or of a solution of sodium acetoacetate (20 mg.) in saline being added to the chloroform and ether preparations. After 2 hr. at room temperature alcohol was added to the latter preparations and the acetylcholine extracted in the usual manner. The results of the assays were: control, 1·3–1·4; chloroform, 6·0; ether, 8·7; chloroform + acetoacetate, 12·5; ether + acetoacetate, 14·5 μg. per g. tissue.

Another specimen of brain tissue was treated with ether-eserine and 20 mg. sodium acetoacetate and incubated for 2 hr. at 37°. Assay showed the presence of 23 μg. acetylcholine per g. extract. The control, treated in the same way except that no acetoacetate was added, gave a yield of about 17 μg. per g.

We have not made extensive examination of nervous tissue from species other than the ox, but it seemed desirable to ascertain if the above values were peculiar to brain tissue from this animal or if values of the same order of magnitude were given by other species. We therefore carried out some preliminary experiments with the rat, this species being selected mainly because the brain could be obtained immediately on removal from the animal. One whole brain was minced and ground in a mortar without any addition. It was then treated with 50 ml. acetone and the extract worked up in the usual manner. Assay showed a content of 0·6 μg. acetylcholine per g. tissue. Another whole brain was similarly prepared, ground with ether-eserine and incubated at 37° for 2 hr.

The yield of acetylcholine in this case was 16 μg. per g. The numerical results from these two experiments, although obtained from different brains, differ so widely in magnitude that they leave little doubt that brain tissue from the rat is quite comparable with that from the ox as regards its production of acetylcholine under the influence of ether.

Formation of acetylcholine in aqueous media

It has previously been shown [Stedman & Stedman, 1937] that when brain tissue is ground with water containing eserine sulphate and the material incubated at 37° little, if any, acetylcholine is formed. Apparently water, like certain organic solvents, destroys the mechanism responsible for the production of acetylcholine in brain tissue. Nevertheless, Mann *et al.* [1938, 2] have found that when a suspension of brain tissue in eserinized phosphate Ringer is shaken at 37° in an O_2 atmosphere, there occurs an accumulation of acetylcholine in the suspension fluid. It thus seemed desirable to compare the amounts of acetylcholine formed under the influence of chloroform with those produced in aqueous media. For the latter experiments, 10 g. portions of brain tissue were ground with a total of 10 ml. phosphate Ringer (*p*H 7·4) or of physiological saline containing eserine sulphate and the suspension shaken in air under various conditions, 100 ml. alcohol being added at the termination of the experiment, after which the acetylcholine was extracted and assayed in the usual manner. The results show that while there is some formation of acetylcholine at room temperature, the amount is small compared with that produced at 37°. At the latter temperature, using a period of 2 hr., the amount produced is generally slightly smaller, but never greater, than that formed under the influence of chloroform at room temperature. If 5 ml. chloroform are added to the suspension in Ringer at the commencement of the experiment, the formation of acetylcholine is inhibited to a considerable degree at 37°. At room temperature, however, it is increased to a value approximately equal to that formed at the same temperature in the presence of chloroform alone. Without discussing these results in detail, it will, we think, be agreed that they can be best interpreted on the assumption that the acetylcholine formed in aqueous media has the same origin as that produced under the influence of chloroform. At room temperature, chloroform is much more efficient than Ringer; at 37°, however, its deleterious action again becomes apparent.

Many experiments have also been carried out with suspensions of brain tissue in phosphate Ringer at 37° to which 20 mg. glucose or of sodium pyruvate have been added. According to Mann *et al.* [1938, 2] the addition of these substrates should considerably enhance the yield of acetylcholine. In some experiments we did, indeed, observe a small but scarcely measurable increase, but in others none was detectable. We were inclined to attribute the discrepancy between our results and those of Mann *et al.* to differences in technique. Our preparations were, for example, shaken in air instead of in O_2. The ox brain tissue which we employed was, moreover, necessarily less fresh than the rat brain tissue used by these authors. We therefore carried out some experiments with rat brains, of which the following are typical.

Four rat brains were finely minced and thoroughly ground in a mortar. Three 1 g. portions of the tissue were separately ground with 0·5 ml. saline containing 0·5 mg. eserine sulphate and washed into flasks with 5 ml. phosphate Ringer (*p*H 7·4). To one flask was added 1 ml. saline and to the remainder 1 ml. saline containing 10 mg. glucose. The flasks were then filled with O_2, placed in a bath at 37°, and shaken for 2 hr. After cooling to room temperature, 0·5 ml.

chloroform was added to one of the flasks containing glucose and the contents vigorously shaken. All were then left for 1 hr. at room temperature, when the experiment was stopped by the addition of 50 ml. acetone to each flask. The acetylcholine was then extracted and assayed in the usual manner. Fig. 4 shows the effect on the leech muscle of equal volumes of extracts from the control (no added glucose) and from the portion to which glucose was added. It is clear from this that, while the yield of acetylcholine from the latter was slightly greater than from the control, the difference is so small as to be of doubtful significance. The same tracing is continued in Fig. 5, from which it is apparent that shaking with chloroform under the conditions used in this experiment causes no increase in the yield of acetylcholine.

Another experiment was carried out which was identical with the above except that the amount of glucose added was restricted to 2 mg. and the treatment of one portion with chloroform omitted. In this experiment a quantitative assay of the yield of acetylcholine was made. This showed that the quantity produced in the experiment with added glucose, which was identical within the limits of experimental error with that produced in its absence, was at least 18 μg. per g.

It should be noted that the above results are in complete contradiction with those of Mann *et al.* [1938, 2] who claim (1) that the addition of glucose to the suspension medium causes a large increase in the yield of acetylcholine, and (2) that treatment with chloroform, as carried out in our experiment, causes a further increase. We are at a loss to explain the discrepancy between the two investigations. Our experiments were carried out under conditions as similar as possible to those of Mann *et al.* They differed merely in the facts that we (1) probably used rather more brain tissue, and (2) extracted the acetylcholine from both the suspension medium and the tissue with acetone instead of assaying that in the suspension medium alone. This method of extraction should not, however, remove acetylcholine from the hypothetical "preformed-precursor", for, as we have shown in our preceding experiments that formed as a result of treatment with chloroform, which is supposed to originate from the "preformed-precursor", is not extractable with acetone until such treatment has been carried out. We should nevertheless feel inclined to attribute our failure to demonstrate any effect of glucose to a difference in technique, were it not for the fact that the yield of acetylcholine which we obtained in the absence of added glucose is of the same order of magnitude as that obtained by Mann *et al.* in its presence. It is probable that the relative amount of suspension fluid which we employed was somewhat smaller than that used by Mann *et al.*, but we cannot think that the discrepancy is attributable to this.

Discussion

The ultimate aim of this investigation is to elucidate the mechanism of the biological formation of acetylcholine. Many of the experiments described above have, however, been carried out with the object of examining certain objections which Mann *et al.* have raised to our view that the acetylcholine formed in brain tissue when such tissue is incubated with chloroform is actually synthesized *in vitro*. Of these objections, the only one for which experimental foundation is claimed is that the yield of acetylcholine from a chloroform preparation is greater at room temperature than at 37°, and that it is, moreover, no greater at the latter temperature than at 0°. As we have shown above, only negligible amounts are, in fact, formed at 0°, but we agree that somewhat more is produced

at room temperature than at 37°. While there is no difficulty in supposing that the mechanism, present in nervous tissue, which is responsible for the synthesis of acetylcholine can function at temperatures of about 18°, as indeed it must so function in cold-blooded animals, it must be admitted that one would expect its efficiency to be greater at the higher temperature of 37°. This minor anomaly has, however, been completely explained by our experiments with ether. It can, we think, scarcely be disputed that the acetylcholine formed in the ether preparation originates from the same source, and by the same mechanism, as that produced in the chloroform preparation. But, as we have shown above, the amount of acetylcholine so formed in the ether preparation is not only greater than that in the corresponding chloroform preparation both at room temperature and at 37°, but the process exhibits a considerably greater efficiency at the higher temperature. It is, we think, clear from this result that the chloroform exerts a deleterious action on the mechanism of the process in question which is much more rapid at 37° than at room temperature.

This explanation of the anomalous temperature effect in the action of chloroform does not, however, entirely dispose of the claim of Mann *et al.* that there exists in brain tissue a "preformed-precursor" of acetylcholine. It is therefore necessary to consider this claim in more detail, for it must be admitted that if the ability of chloroform and, presumably, ether to increase the amount of extractable acetylcholine in brain tissue is merely due, as these authors suggest, to their power of releasing such acetylcholine from a precursor in which it exists in a preformed condition, then experiments such as we have carried out must necessarily be incapable of providing information regarding the mechanism of the biological formation of acetylcholine.

What, then, is the evidence for the existence of the "preformed-precursor" of Mann *et al.*? Briefly, their claim is based largely on the following observations: (1) When a suspension of brain tissue in certain aqueous media is shaken at 37° in O_2, there occurs an increase in the acetylcholine content of the suspension medium. (2) If, after such increase has occurred, the suspension is shaken with chloroform or brought to *p*H 3·0 and left at room temperature for a period of from 30 to 90 min., a further increase takes place. The second increase is supposed to demonstrate the existence, and to be a measure of the amount, of the "preformed-precursor" in the tissue after incubation. Now, it is to be noted that Mann *et al.* only measure the acetylcholine content of the suspension medium. According to Loewi, the aqueous media which they employ are incapable of completely extracting this substance from nervous tissue, and one is therefore justified in concluding that there exists a residue of unextracted acetylcholine in their material. Loewi has further shown that aqueous acids, as well as alcoholic HCl, liberate this "bound" acetylcholine. It is therefore not surprising that the yield of acetylcholine in the suspension fluid slowly increases after acidification. So far the "preformed-precursor" of Mann *et al.* appears to be nothing else than the "bound" acetylcholine of Loewi. But Mann *et al.*, utilizing under somewhat different conditions our observation that treatment of brain tissue with chloroform causes a large increase in its content of acetylcholine, claim to have demonstrated by this means the existence of their "preformed-precursor" and simultaneously to have proved that the acetylcholine produced in our experiments had not been synthesized *in vitro* but had pre-existed in the tissue. While it has not been definitely proved, it must be presumed, unless evidence to the contrary is provided, that the additional acetylcholine formed under the influence of chloroform in the experiments of Mann *et al.* has the same origin as that produced by the same agent under the

conditions which we employ. If, however, this presumption be accepted, it is clear that the source of this acetylcholine cannot be the "bound" acetylcholine of Loewi, for our experiments have proved without doubt that treatment of brain tissue with chloroform causes the formation in such tissue of an amount of acetylcholine many times greater than can be extracted from similar but untreated tissue by solvents which remove Loewi's "bound" acetylcholine, and we are thus faced with the prospect of dealing with the presence of two different bound forms of the substance. Actually, however, there is at present no evidence for the existence of the "preformed-precursor" as distinct from Loewi's "bound" form. Its conception is merely an interpretation of our discovery of the influence of chloroform. Mann *et al.*, in fact, virtually argue that its presence is proved by the ability of chloroform to cause the formation of acetylcholine in brain tissue, and hence that such acetylcholine must originate from it.

The quantitative data which we have provided give, moreover, no evidence in support of the existence of the "preformed-precursor". Thus, minced and ground ox brain which we use in our experiments contains a total, including Loewi's "bound" acetylcholine, of about 1·5 μg. acetylcholine per g. Similar tissue from a rat's brain contains even less. Yet by incubating such tissue with ether, the effect of which must clearly be similar to that of chloroform, these amounts can be raised to 17 and 16 μg. per g. for the ox and rat respectively, figures which are of the same order of magnitude as those obtained by Mann *et al.* in experiments in which they claim that a considerable synthesis of acetylcholine has occurred. The conclusion thus seems inescapable that the acetylcholine formed in the presence of certain organic solvents has the same origin as that formed in aqueous media, i.e. that both are synthesized *in vitro*. We do not yet know the complete function of the organic solvent. One of its functions is, of course, to act as a vehicle for conveying the eserine into the tissue, but it probably also exerts some other action.

If it be agreed, as we think it must be, that the acetylcholine formed in the presence of chloroform or ether is synthesized *in vitro* and does not exist in a preformed condition in the tissue, it follows that any process capable of modifying the yield formed under these conditions can be regarded as legitimate evidence concerning the mechanism of the synthetic process. Now, with the exception of choline itself, which has not given entirely consistent results, we have so far discovered only two substances which modify the process to a considerable extent. These are sodium pyruvate and sodium acetoacetate. The former substance, when added to our chloroform preparation, produces a considerable inhibition of the formation of acetylcholine both at room temperature and at 37°. We conclude that sodium pyruvate cannot be a precursor of acetylcholine, and we might here also point out that its behaviour is scarcely consistent with the view that the acetylcholine formed in our chloroform preparation originates from a "preformed-precursor". Sodium acetoacetate, on the other hand, considerably increases the yield of acetylcholine both in the chloroform and ether preparation, the amount of additional acetylcholine for which it is responsible representing 4–5 times that present in the minced tissue before treatment with the solvent. The combined effect of incubation, after addition of ether-eserine, and addition of sodium acetoacetate is much greater than this and, as we have shown, is capable of raising the acetylcholine content to 23 μg. per g., i.e. to about 15 times that originally present. We consider this to constitute substantial evidence that acetoacetic acid is, in fact, a precursor of acetylcholine. We admit that we should like to confirm this view by experiments with aqueous media, but we have so far failed to realize the necessary conditions. The second

precursor must, of course, be either choline itself or some derivative of choline. We do not propose, however, to discuss this point at present since, as pointed out above, our experiments with choline have not been entirely consistent.

Summary

A study has been made of the formation of acetylcholine by brain tissue *in vitro* under various conditions. The amount formed when a suspension of minced ox brain in eserinized Ringer is shaken for 2 hr. at 37° is approximately equal to that produced by grinding the minced tissue with chloroform-eserine and keeping it at room temperature for the same period.

Ether resembles chloroform in its ability to promote the formation of acetylcholine in brain tissue, but it is more efficient. In the case of ox brain, increases from 1·5 μg. before to 17 μg. per g. after treatment have been observed. The corresponding figures for rat brain tissue are 0·6 μg. and 16 μg. respectively.

The effect of ether is greater at 37° than at room temperature. The reverse holds for chloroform. An explanation of this difference is offered. Contrary to the statement of Mann *et al.* the amount of acetylcholine formed in the chloroform preparation at 0° is exceedingly small and is almost negligible compared with that produced at 37°.

Addition of sodium acetoacetate to the chloroform or ether preparations increases the yield of acetylcholine. Contrary to the statement of Mann *et al.* this increase also occurs in the chloroform preparation at room temperature.

When a suspension of minced rat brain in eserinized Ringer is shaken in O_2 for 2 hr., acetylcholine to the extent of 18 μg. per g. is formed. Addition of glucose to the suspension medium under conditions which have been carefully defined does not appreciably increase the yield. These results are contrary to those of Mann *et al.*

It is suggested (1) that acetoacetic acid is a precursor of acetylcholine, and (2) that the "preformed-precursor" of Mann *et al.* does not exist.

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